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Ectomycorrhiza Formation of *Tricholoma matsutake* on Mature Pine Root in Situ

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Abstract: We studied on the ectomycorrhiza formation of *Tricholoma matsutake* on mature pine (*Pinus densiflora*) root using matsutake solid spawn in situ. To find out suitable medium for the solid spawn, the matsutake mycelium growths on different media were compared. Among them, GYP medium (glucose 20g, yeast extract 5g, peptone 5g, KH_2PO_4 1g, $CaCl_2$ 50mg and vitamin B_1 50mg) and GWR medium (glucose 20g, wheat bran 50g, rice bran 50g, KH_2PO_4 1g, $CaCl_2$ 50mg and vitamin B_1 50mg) showed good proliferation of matsutake mycelium. The GYP and GWR media were used to make charcoal and vermiculite-soil spawns. The ergosterol amounts of charcoal and vermiculite-soil spawns were 1.53 and 1.61 times higher than that of the natural shiro, respectively. In spring, the roots of about 30-year-old red pine were shortened with unsterilized scissors. About twelve months later, the regenerated root tips from shortened points were inoculated with charcoal and vermiculite-soil spawns, respectively. After four months by the inoculation with charcoal and vermiculite-soil spawns, 55% and 50% of experimental plots inoculated with charcoal and vermiculite-soil spawns revealed dichotomic root tips characteristic to ectomycorrhiza, respectively. The nested PCR amplification results confirmed that the dichotomous root tips were colonised by matsutake spawn. Our findings demonstrated that the ectomycorrhiza of *T. matsutake* could be formed on the mature pine root in situ by using matsutake solid spawns.

Keywords: *Tricholoma matsutake*, Ectomycorrhiza, Solid Spawn, Inoculation, *Pinus desiflora*, Nested PCR

1. Introduction

Tricholoma matsutake, a member of the family Tricholomataceae, order Agaricales, has been reported from Korean peninsula, China, Japan and Bhutan, meanwhile *Tricholoma matsutake* in Korean peninsula, Japan, and northeastern China are closely related, but distinct from those in southwestern China and Bhutan [1]. In DPR Korea, the fruiting bodies of *T. matsutake* occur in almost areas including Mt. Myohyang, Mt. Kumgang, Buryong, Yangdok, Myongchen, Kimchaek, Kilju, Rason, Chosan etc.

T. matsutake usually occurs in Japanese red pine (*Pinus densiflora*) forests and also under trees of *Pinus thunbergii*, *Pinus pumila*, *Tsuga diversifolia*, *Tsuga sieboldii*, and *Abies veitchi* [2].

Matsutakes appear on nutrient-poor soils, such as granite, chert, and sandstone, which are acidic (pH 4.5-5.0) and develops special structure called “shiros” in symbiotic relation with host root [3]. Shiros are mycelial aggregations that develop in association with the roots of host trees and soil particles in well drained and nutrient-poor forest soil, from which matsutake generates. The fruiting bodies of *T. matsutake* occur on the zone of around 25 cm inwards from the growing tip of the shiro, which is called an “active mycorrhizal zone” [4]. Shiros expand at a rate of 10~15 cm per year or 17 cm per year [5].

The matsutake has been known as one of the most expensive edible and medicinal mushroom in many countries

including Japan, but in spite of more than 100 years researches, its artificial cultivation method has not been availible [6]. The main reasons are that *T. matsutake* forms a symbiotic association with the roots of living host trees and receives nutrients from host, but possesses little ability to decompose organic matter [7-9].

For the production of fruiting body of *T. matsutake* as ectomycorrhizal (EM) fungus, it is necessary to create mycorrhizal association with host root, so that the artificial formation of ectomycorrhiza of matsutake with pine root is the key step for the establishment of matsutake cultivation technology. In 1999, it was reported that a fungal mantle and Hartig net developed on roots of *Pinus densiflora* seedlings cultivated on sterile vermiculite with nutrient solution after inoculation with *T. matsutake* culture [10].

This was the first report of the formation of a typical ectomycorrhiza with host root by *T. matsutake*. Researches had been carried out for in vitro ectomycorrhizal infection on *Pinus densiflora* roots by *T. matsutake* using sterilised soil or artificial substrate [11-13]. Growth stimulation of a shiro-like, mycorrhiza forming, mycelium of *Tricholoma matsutake* on solid substrates by using non-ionic surfactants or vegetable oil has been investigated [14]. Many different approaches, including spraying of spores on the ground of pine forests, inoculating cultured mycelia into the soil, and planting trees infected with *T. matsutake*, showed that no growth of *T. matsutake* was observed during these trials with the exception of planting trees infected with *T. matsutake* [15, 16]. In 2005, for the first time, the root of mature pine tree was successfully inoculated with the mycelial pieces of *T. matsutake* in a sterilized substrate [17, 18]. However, there was a report that mass liguid inoculum could be used as matsutake inoculum for artificial inoculation of pine forest under the appropriate environmental conditions [18]. To the best of our knowledge, it seems that there was no report for the formation of ectomycorrhiza on the mature pine root with matsutake spawn cultivated in inorganic substrate under natural condition.

2. Materials and Methods

2.1. Study Sites

This study was conducted in HaeYon-ri, Kimchaek City, North Hamgyong province and in Gosang-ri, Yangdok county, South Pyongan province, DPR Korea (Table 1). Study sites were dominated with 20~30-year-old *P. densilflora* trees and most conditions including soil properties and pH were primarily coincident with that of development of the matsutake. Furthermore, there was no record of matsutake fruit body development during last 2 decades. The dominant specieswas *Pinus densiflora*, and there were some *Quercus acutissima*, *Rhododendron mucronulatum*, *Fraxinus rhynchophylla* sparsely.

2.2. Preparation of T. matsutake Isolates

Matsutake isolates were obtained from the fruit body of *T. matsutake* collected from the study site in 2018 and stored on slants of Hamada's agar medium [19] at 4°C until use.

Morphological characteristics were observed after incubation for 45 days and DNA identification of *T. matsutake* was carried out using matsutake-specific primers TmF/TmR [20].

2.3. Selection of Optimum Medium

Eleven different agar media were used to investigate the mycelial growth of *T. matsutake* (Table 2). The different kinds of slants were made and sterilized at 121°C for 20 min. Mycelial slices were cut from colonies of matsutake isolates and inoculated on the center of slants. After inoculation, the test tubes were incubated at 23°C for about 45 days and measured the colony size.

Table 1. *Study sites conducted in the experiments.*

Sites	Location	Average age of pine	Density of tree (tree/ha)	Soil	pH	Sea level (m)	Orientation	Average annual precipitation
HaeYeon-ri	E40°35′ N129°07′	20	1100	granite	5.2	150	South-west	791.6
GeSang-ri	E39°18′ N126°50′	30	1200	granite	5.5	450	South-west	1,037.8

Table 2. *Compositions of media used in the experiment.*

Composition	Medium (g/L)										
	HA	PDA	GYP	YPP	YPR	YR	GY	YRB	YP	GPP	GWR
Potato		200									
Glucose	20	20	20	20	20	20	20	20	20	20	20
Yeast extract	3		5	3	5	5	3	3	3	5	2
Peptone			5	3	5	5			3	5	
$MgSO_4$	0.5						0.5	0.5			
KH_2PO_4	1		1		1		1	1	1	1	1
$CaCl_2$			0.05	0.05							0.05
Vitamin B1			0.05								0.05
Vitamin PP				0.05						0.05	
Pine pollen					0.1		0.1		0.1		
Rice bran					20	20		20	20		50
Wheat bran											50
Agar	20	20	20	20	20	20	20	20	20	20	20

2.4. Spawn Culture of Matsutake Isolates

Charcoal substrates with ca. 5mm diameter were treated with hydrochloric acid to make it's pH to 5.5. Then the GYP medium was added into charcoal substrate up to 35% humidity, and the matsutake isolate was inoculated into charcoal substrate and incubated at 23°Cfor 40 ~ 50 days to make charcoal spawn. A vermiculite-soil spawn was made as follows: GWR medium was added into vermiculite-soil substrate (5: 5, v/v) up to 35% humidity, and the substrate was inoculated by the charcoal spawn and incubated at 23°C for 60 days. Ergosterol amounts of matsutake spawns and natural shiro were measured by the method of Zhao et al. [21].

2.5. Inoculatoin of T. matsutake Spawn on Mature Pine Root

In spring, the roots (ca. 3~5mm in diameter) of about 30-year-old pines were shortened with scissors in 10~20cm depth of soil, and buried with fallen leaves and soil. About twelve months later, the regenerated roots from shortened points were sterilized with 75% alcohol and inoculated with charcoal and vermiculite-soil spawns, respectively. For the prevention of any native EM fungi, the inoculation portions were wrapped up with the riversand or 20cm-depth mineral soil and covered with a perforated vinyl sheet.

Inoculation of matsutake spawns were carried out in spring when soil temperature was about 10°C and the soil native microbes did not flourish.

2.6. Morphological and Molecular Analysis of Ectomycorrhiza Formation by Matsutake Spawns

Inoculated root portions were excavated cautiously four months later, and primarily observed if there were dichotomic root tips and matsutake odor from root. Dichotomic root tips with matsutake odor were collected and observed under a dissecting microscope.

A nested PCR amplification was carried out on DNA extracts (CTAB method) from samples (about 15mg). Amplification using fungal-specific primers ITS1/ITS4 [22] was followed by reamplification using matsutake-specific primers TmF/TmR [20]. The first amplification with primers ITS1 and ITS4 was carried out in a 25 μl reaction solution including 2.5 μl 10× PCR buffer, 200 μM of each dNTP, 1 μM of each primer, 1.5U Taq polymerase and 1 μl template DNA. Reactions were run in a C1000 TouchTM Thermal Cycler. The thermal cycling was followed by an initial denaturation at 94°C for 10 min, 35 cycles of denaturation at 94°C for 40s, annealing at 55°C for 40s and extension at 72°C for 1min, and then a final extension at 72°C for 10 min. The product of the first amplification was diluted 10 times with sterilized deionized water and 1.0 μl of the diluted solution was used as the template for the nested PCR. Conditions for the nested PCR using primers TmF and TmR were similar to the first PCR, except for an initial denaturation at 95°C for 10 min and denaturation at 95°C for 40s. The PCR products were detected by electrophoresis in 0.8% (w/v) agarose gel supplemented with ethidium bromide. The DNA was visualized and photographed over a UV light source. A DM 2 000 molecular weight marker (CWBIO, China) was used as the size standard.

2.7. Statistical Analysis

Statistical analysis was carried out in SPSS 22.0. Significant differences (mean ± SE) among treatments were compared by Tukey's HSD test at $P < 0.05$.

3. Results

3.1. T. matsutake Isolates

Morphological characteristics of isolates on slants of Hamada's agar medium showed the typical characters of *T. matsutake* (Figure 1) and molecular analysis using matsutake-specific primers TmF/TmR also proved the isolates were *T. matsutake* (Figure 6).

3.2. Selection of Optimum Medium

For the selection of optimum medium, matsutake isolates were grown in 11 different media including Hamada's agar media. Among them, GYP and GWR media showed good proliferation of matsutake mycelium with day growth rate of 0.46±0.03, 0.53±0.01 mm/d, respectively (Figure 2). The growth rates of *T. matsutake*on GYP and GWR media were 1.27 and 1.47 times higher than that on Hamada medium known as common matsutake medium.

3.3. Spawn Culture of Matsutake Isolates

The mycelial density of charcoal spawn and vermiculite-soil spawns were higher than that of the natural shiro and all the spawns also had the odor of *T. matsutake* (Table 3, Figure 3).

The ergosterol amounts of charcoal and vermiculite-soil spawns were 110.5±4.3, 116.1±5.8 μg/g and 1.53, 1.61 times higher than that (72.1±3.2μg/g) of the natural shiro, respectively (Figure 4).

In spring, the roots (ca. 3~5mm in diameter) of about 30-year-old red pines were shortened. About twelve months later, the regenerated root tips from shortened points (Figure 5. A, B) were inoculated by spawns. Inoculation was carried out in May when the soil temperature was about 9~13°C.

Four months later after inoculation, the inoculated portions were examined. The dichotomic root tips, characteristic to ectomycorrhiza, were formed in the inoculated root portions (Figure 5C, D, E).

DNA analysis confirmed the presence of *T. matsutake* in matsutake inoculated roots (Figure 6). No matsutake-specific bands were amplified from root samples of non-inoculated root portions as control. More than 50% of samples showed the dichotomic root tips characteristic to putative matsutake mycorrhiza, and the nested PCR results from DNA extracts

of any sample were also positive (Table 4).

We found that charcoal and vermiculite-soil spawns could be used as matsutake inoculum on the mature pine roots, but we consider the results must be confirmed in the future with the development of matsutake fruiting body.

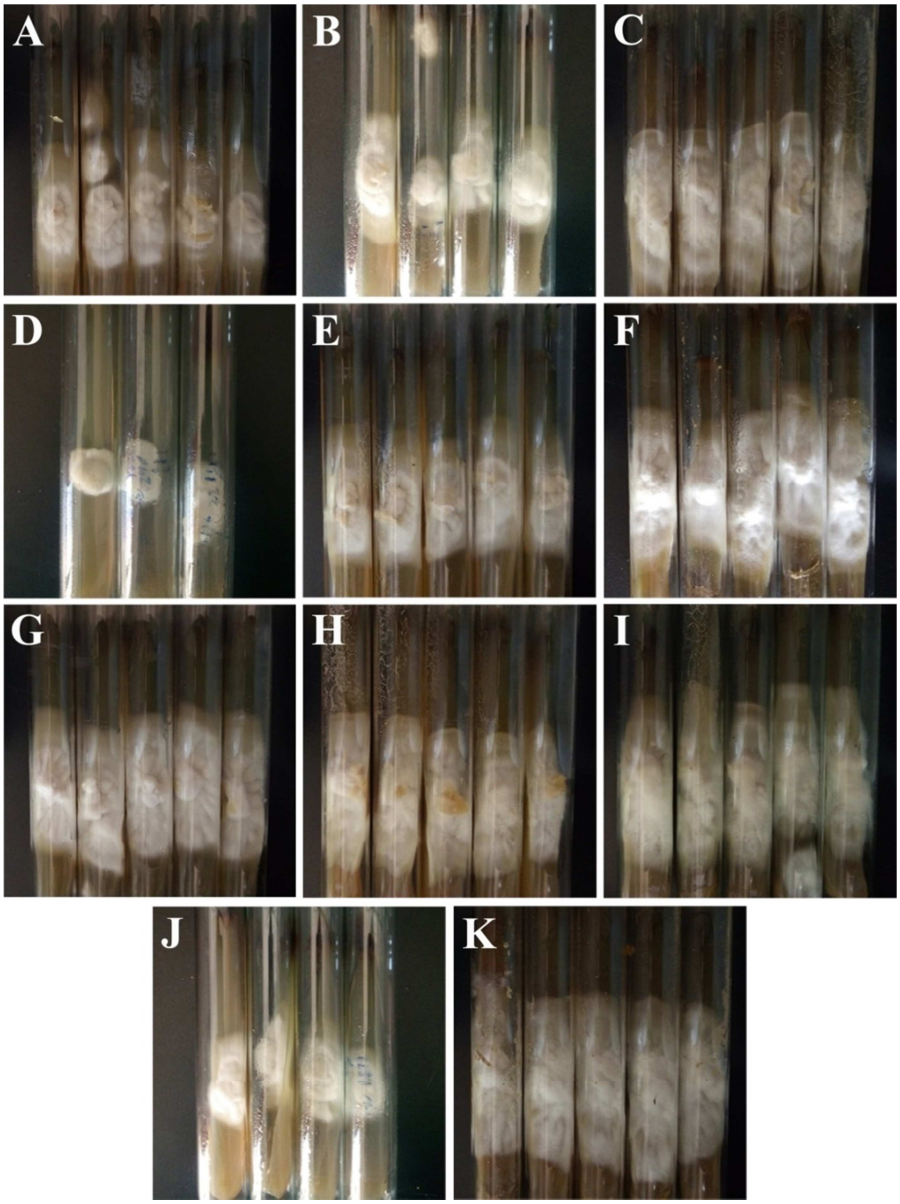

Figure 1. *T. matsutake grown on different kinds of media. A: HA, B: PDA, C: GYP, D: YPP, E: YPR, F: YR, G: GY, H: YRB, I: YP, J: GPP, K: GWR.*

Table 3. *Culture properties of spawns.*

Spawns	Germination (d)	Spawn running (d)	Hyphal density[1)]	Odor[2)]
Charcoal spawn	10	50	+	+
Vermiculite-soil spawn	7	45	+	+

[1)]: + higher than natural shiro. [2)]: + matsutake odor.

Table 4. *Examination of inoculated sites in 2021 yr.*

Sites (spawn)	Inoculation		Examination			
	Date	Number of trial	Date	Number of examination	Number of dichotomic root-site	Molecular analysis
HaeYeon-ri (vermiculite-soil spawn)	2021.5	30	2021.9	14	7	+
	2021.5	15	2021.9	8	4	+
GeSang-ri (charcoal spawn)	2021.5	40	2021.9	20	11	+

＋ positive in nested PCR amplification

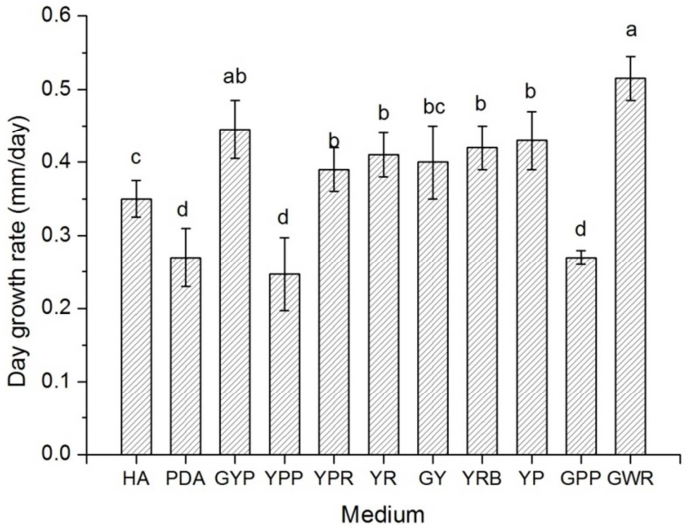

Figure 2. *Effects of various media on mycelial growth of Tricholoma matsutake. Bars without shared letters indicate significant difference at P < 0.05.*

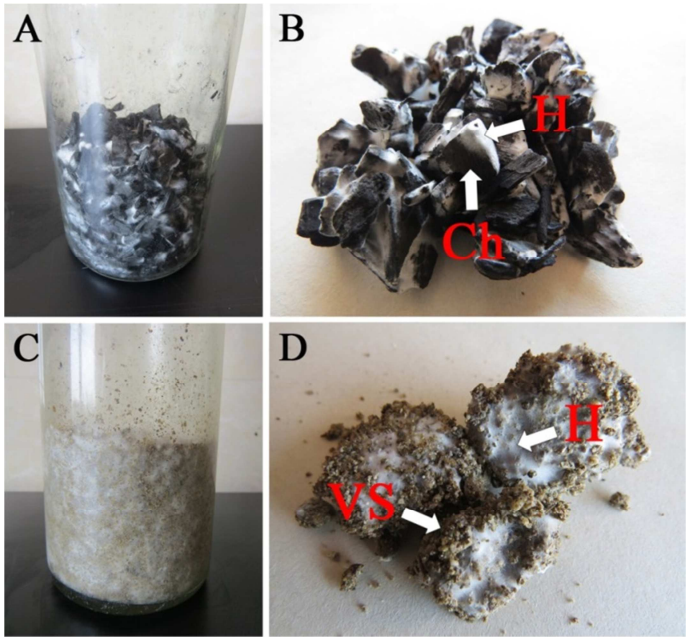

Figure 3. *Charcoal spawn and vermiculite-soil spawn. A: Charcoal spawn in bottle, B: Charcoal spawn used in inoculation, C: Vermiculite-soil spawn in bottle, D: Vermiculite-soil spawn used in inoculation. Abbreviations, H: matsutake hyphae, Ch: charcoal, VS: vermiculite-soil.*

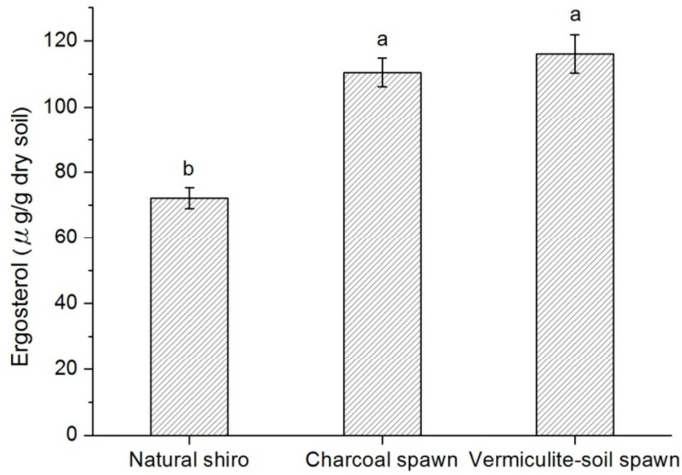

Figure 4. *The amounts of ergosterol in natural shiro and spawns. Bars without shared letters indicate significant difference at P < 0.05.*

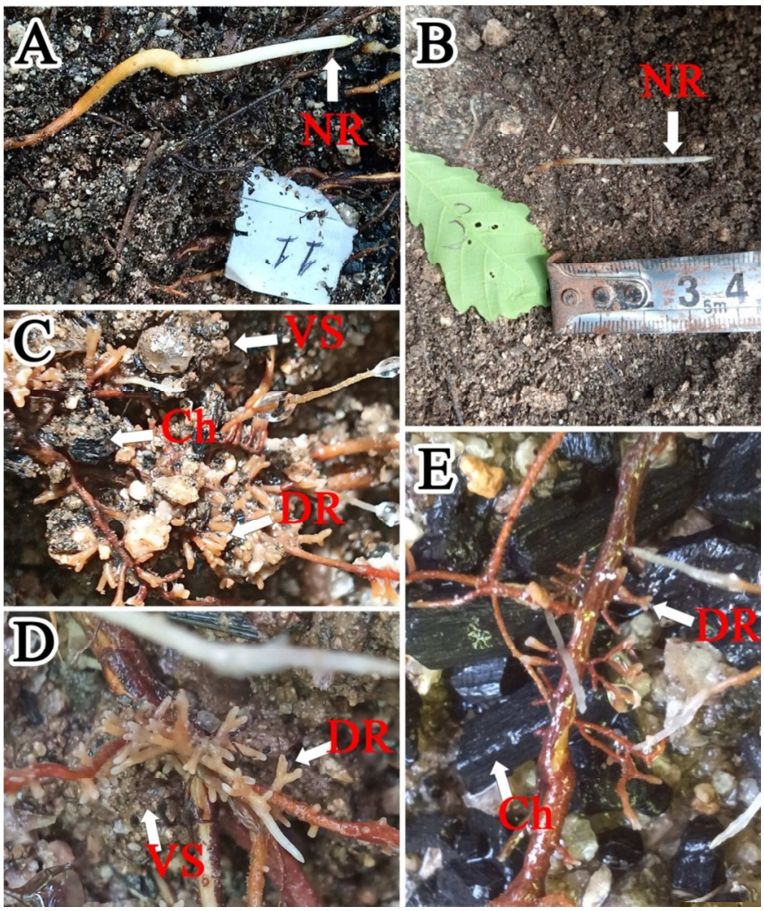

Figure 5. *New generated root tips (NR) and dichotomic root tips (DR) observed in inoculated sites. A and B: New generated root tips from shortened points, C: Dichotomic root tips in charcoal (Ch) and vermiculite-soil spawn (VS) inoculated sites, D: Dichotomic root tips in vermiculite-soil spawninoculated sites, E: Dichotomic root tips in charcoal spawninoculated sites.*

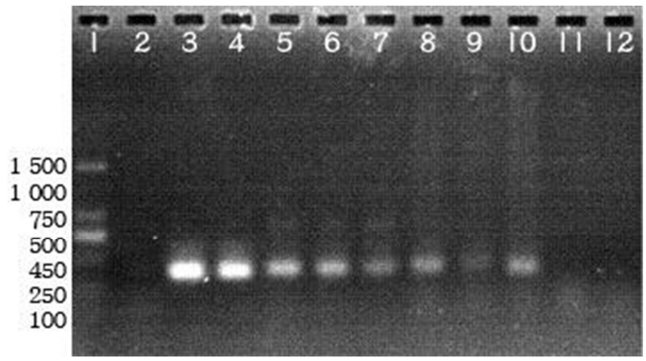

Figure 6. *Nested PCR on DNA extracts from inoculated root samples. Second step reamplification of the samples, using the matsutake specific primer pair TmF/TmR. (1: DNA maker, 2: Negative control, 3: Fruiting body of T. matsutake, 4: T. matsutake isolates, 5: Natural shiro mycelium, 6: Natural shiro root, 7 and 8: Charcoal spawn inoculated root, 9, 10: Vermiculite-soil inoculated root, 11 and 12: control).*

4. Conclusion

T. matsutake is a symbiotic ectomycorrhizal fungus which is mainly produced on root of pine tree [6]. The growth of *T. matsutake*, which uses symbiotic nutrition, is very slow on artificial media [18], so that it is necessary to examine the effects of various nutrients on the matsutake mycelial growth in order to produce matsutake spawns. Although many nutrients have good effects on matsutake mycelial growth [23], we found that the media including yeast extracts, wheat bran, rice bran, and vitamin B_1 gave the best mycelial growth for preparation of matsutake spawns.

Many studies were carried out to form artificial matsutake ectomycorrhiza on pine roots [17, 18]. Yamada et al. inoculated matsutake isolates on 1-wk-old seedlings, and demonstrated that the fungus formed a sheath and Hartig net on the pine lateral roots to form successfully matsutake ectomycorrhiza during three months after inoculation [10]. This result indicates that cultured *T. matsutake* mycelium can form true ectomycorrhizas with *P. densiflora* seedlings. Especially, Guerin-Laguette et al. regenerated mycorrhiza-free short roots in 50-year-old *Pinus densiflora* trees and inoculated mycelial pieces of *T. matsutake* on mycorrhiza-free short roots [17]. After four-and-a-half months, the matsutake–inoculated-roots were colonized by matsutake mycelia. Although this study was limited to a single tree, the results clearly confirm the symbiotic infection of mature *P. densiflora* roots by matsutake isolates. In order to produce fruiting bodies of *T. matsutake*, mass liquid inoculum of matsutake isolates were inoculated on pine forest ground and after one year of inoculation, no external difference was observed between the artificially inoculated mycelia and the

naturally existing mycelia of *T. matsutake* [18].

In their study, the results of artificial *T. matsutake* development have not been reported but they suggested that using liquid inoculum of *T. matsutake*, used in their research, may be new technique to obtain artificial *T. matsutake*. *T. matsutake* is an obligate fungus that forms a symbiotic association with the roots of living pine trees and receives nutrients from them [7-9], so thatit is very important to produce spawn to form artificial ectomycorrhiza on the roots of mature pine for the cultivation of *T. matsutake* in pine forest. Previous studies on ectomycorrhiza formation of matsutake in pine root mostly used matsutake mycelia on agar media or liquid medium as inoculum [10, 17, 18].

We predicted that the inoculum with nutrient-poor substrates such as charcoal, vermiculite and mineral soil would be favorable to mycorrhiza formation in situ, because spawn with nutrient-rich substrates could be decayed in the soil for months to damage the host roots inoculated with matustake spawn. Our results indicate that *T. matsutake* spawns cultivated in charcoal or vermiculite-soil can form ectomycorrhizas with mature pine roots in situ.

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